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Molecular characterization, expression and functional analysis of a nuclear oligomerization domain proteins subfamily C (NLRC) in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Pattern recognition receptors (PRRs) are involved in the effective innate defense against several microbes. Here, we identified a nucleotide-oligomerization domain (NOD)-like receptor subfamily C (NLRC) from Japanese flounder (*Paralichthys olivaceus*). Full-length transcript of JfNLRC is composed of 3976 bp encoding a protein of 1175 deduced amino acid residues. The presence of a signature nucleotide-binding domain (NACHT) and leucine-rich repeated domain (LRR) suggested that the protein is a member of the NLR family. Interestingly, its C-terminus presents an extra PRY/SPRY (B30.2) domain similar to fish in the Trim (finTrim) family. A phylogenic tree of JfNLRC revealed that full-length JfNLRC diverged from the NOD1 and NOD2 clusters, and the NACHT domain in JfNLRC was clustered within the NLRC3 group. Stimulation by formalin-killed *Edwardsiella tarda*, *Streptococcus iniae*, and lipopolysaccharide (LPS) showed that the JfNLRC expression was raised a few hours after stimulation, suggesting this novel protein is involved in the immediate response against both Gram-positive and Gram-negative bacteria. Furthermore, the IL-1β mRNA expression level in JfNLRC-over-expressing HINAE cells was significantly increased, when compared to a control, after LPS-stimulation and *E. tarda* infection. These results suggested that JfNLRC probably induced IL-1β gene expression mediated by LPS-stimulation.

1. Introduction

Among fish and higher eukaryotes, the innate immune system is used as the first line of defense to combat various types of pathogens. Three kinds of germline-encoded pattern recognition receptors (PRRs) have been implicated in pathogen control and elimination. These include the Toll-like receptors (TLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs; alternatively called Nucleotide-binding domain and leucine-rich repeat containing family Receptors), and the retinoic acid-inducible gene (RIG-1)-like receptors (RLRs). Individual PRRs recognize pathogen-associated molecular patterns (PAMPs) from all major classes of microbes, including bacteria, viruses, yeast and parasites. PAMPs are uniquely expressed by microorganisms and have highly conserved structures. Some common PAMPs are lipopolysaccharide (LPS), peptidoglycan, DNA/RNA of virus and bacteria [1].

NLRs are the intracellular receptors implicated in innate recognition and defense against bacteria. A common characteristic of NLR proteins is the presence of a central nucleotide-binding domain (NB), which is flanked by highly variable leucine-rich repeat (LRR) domains, which are also called NB- and LRR-containing (NLR) sensors [2]. The NB domain is alternatively called NACHT domain, *which can be found in* proteins such as NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*), and TP1 (telomerase-associated protein). The proteins containing this NACHT domain are classified in several subgroups according to the N-terminal effecter domain: 1) CARD-containing NODs, IPAF, and CIITA; 2) PYRIN-containing NALPs; and 3) BIR-containing NAIP [3,4]. NLRs detect and sense microbial components

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by binding its central NB and C-terminal LRR to the microbial components that then lead to the activation of inflammasomes and promote the secretion of cytokines [5–7].

Recently, a number of fish NLRs have been characterized from zebrafish [8], channel catfish [9] and grass carp [10]. Laing and colleagues [8] classified NLRs into 3 subfamilies (subfamilies NLR-A, NLR-B and NLR-C) according to their domain organization. NLR subfamily C (NLRC) is unique in that the N-termini or C-termini of its members possess an additional domain that includes a pyrin (P) and an uncharacterized (X) domain at N-termini, and a B30.2 (PRY/SPRY) domain at C-terminus. The B30.2 domain is a combination of Serine–Proline–Arginine–Tyrosine (SPRY) and Proline–Arginine–Tyrosine (PRY) regions, *i.e.*, a single SPRY region and a single PRY region (approximately 170 amino acids), which play an important role in innate immunity. The B30.2 domain is also found in some tripartite motif (Trim) proteins, which have been implicated in virus infection [11,12]. Teleost-specific (finfish-specific) Trim (finTrim) has been identified and is thought to have emerged from the duplication and positive selection of Trim [13]. Since a number of fish NLRCs are similar to other NLR, teleost NLRCs may have evolved to have a role in innate immunity.

Here, we cloned the complete cDNA sequence of a novel NLRC from Japanese flounder (*Paralichthys olivaceus*) (JfNLRC), which harbors a B30.2 domain. We showed the JfNLRC gene expression in the tissues stimulated with formalin-killed bacteria and lipopolysaccharide (LPS). We also showed the functional responses to inflammatory cytokine expression in JfNLRC-over-expressing cells.

2. Material and methods

2.1. Sample preparation and bacterial stimulation

For the experimental fish, Japanese flounder (*P. olivaceus*) weighing about 250 g were used for synthesis of a cDNA template for RACE PCR, while those weighing about 4 g were used for RT-PCR analyses. The bacteria strains, *Streptococcus iniae* strain 02 [14] and *Edwardsiella tarda* strain NE8003 [15], used for immunostimulation, were treated with formalin as described previously [16]. Briefly, *S. iniae* was cultured in Todd-Hewitt medium (Becton, Dickinson and company, Franklin Lakes, NJ, USA) supplemented with 1% glucose and incubated at 26 °C for 24 h with gentle agitation while *E. tarda* was cultured in LB broth at 37 °C for 24 h with shaking. Bacterial cells were collected, re-suspended in 1 ml phosphate buffered saline (PBS) and counted by making a 10-fold dilution of the whole cell suspension. Formalin (0.5%) was subsequently added to the bacterial cell suspension and the suspension was incubated at 37 °C for 2 h then at 4 °C for 3 h. Cells were washed three times with PBS and re-suspended in 1 ml PBS. For the challenge test, fish were acclimatized at 20 °C in a 120 l tank for 7 days before being injected with 100 μl of formalin-killed cells (FKCs) of *S. iniae* (4×10^8 CFU/ml) and *E. tarda* (2×10^8 CFU/ml). PBS (100 μl) was used as a negative control. Head kidneys of infected fish were collected at 3, 6 and 12 h post-challenge.

2.2. Leukocyte isolations and cell-stimulation with LPS

Leukocytes were isolated from flounder whole kidney and peripheral blood by Percoll gradients according to a previous study [17]. Briefly, peripheral blood or the cells from the whole kidney were diluted 1:10 with Leibovitz's L-15 medium (Invitrogen Life Technologies) and supplemented with 10 units/ml of heparin, 10 mM of HEPES, 60 mM of NaCl, 5% fetal bovine serum (FBS) (Invitrogen Life Technologies), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 250 ng/ml of amphotericin B. The leukocytes were separated with a 51% iso-osmotic Percoll (GE healthcare UK Ltd, Buckinghamshire, England) gradients. The cells were re-suspended in the cold L-15 medium and used for cell separation.

For stimulation experiments, 1×10^6 leukocytes and 1×10^6 cells HINAE (Hirame natural embryos) cells were cultured in a 24-well plate with 250 μg/ml of LPS (*Escherichia coli* serotype 0111:B4) (Sigma, St Louis, MO) at 20 °C for 6, 24 and 48 h for the leukocytes and at 25 °C for 24 and 48 h for HINAE cells. The harvested leukocytes were centrifuged at 1600 rpm (approximately 200 rcf) for 5 min at 4 °C, and re-suspended in RLT plus reagent provided by the RNeasy mini kit (Qiagen) for RNA extraction.

2.3. RNA extraction and cDNA synthesis

To determine the tissue distribution of JfNLRC, various tissues [brain, eye, gills, kidney, heart, intestine, liver, muscle, peripheral blood leukocytes (PBL), skin, spleen and stomach] from an apparently healthy fish and head kidney of a fish that had been challenged with FKCs of *S. iniae* or *E. tarda* were collected. Total RNA was extracted from the tissues using RNAiso (Takara Bio Inc, Otsu, Japan) according to manufacturer's instructions. First-strand cDNA synthesis was performed with 1 mg of total RNA and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Three fish for each treatment were used to analyze gene expression. Total RNA from leukocytes and HINAE cells was extracted with an RNeasy mini kit (Qiagen), and the cDNAs were synthesized with the SuperScript™ III First Strand synthesis system (Life Technologies) after removing the contaminated genomic DNA with DNase I (Life Technologies).

2.4. Identification of the Japanese flounder NOD-like receptor cDNA

To identify complete cDNA sequence, RACE-PCR analysis was conducted by using SMART™ RACE cDNA amplification kit (Takara Bio Inc.). The sequence of specific primer sets Nacht5Race1 (5′-GAGGAAGTCGACACTCGTCCAGACCGTCGAAG-3′), Nacht5Race2 (5′-CCCTCAGCAAATTCAGCTCTCGGAACGCAATGC-3′), Nacht5Race3 (5′-CTCGTGGACGTTTGTGGTCACTCTCCTGC-3′) and Nacht5Race4 (5′-GATCGGTGGCTCCATAGTAGCTGTTGCTGCATCG-3′) were designed to identify 5′-RACE products whereas Nacht3Race1 (5′-AGAGGAGCTGAGGCAGATCCCCTCTGGAG-3′), Nacht3Race2 (5′-GTGCGGCATCGATATCAGAGCCGCCTCAGTTTACTC-3′), Nacht3Race3 (5′-CGGAGAGGAGCTGTGAAGCTCTGTCGTC-3′) and Nacht3Race4 (5′-GAGAGATCTGGACCTGAGCAACAACGACCTGCAG-3′) were used to identify 3′-RACE products. The PCR profile consisted of 5 min at 95 °C followed by 30 cycles of 95 °C 30 s, 50 °C 30 s and 72 °C 2 min, followed by final extension at 72 °C 10 min. All RACE-PCR products were cloned into pGEM® T-Easy vector (Promega KK, Tokyo, Japan) and subjected to automated sequencing in both directions.

2.5. Sequence analysis

Protein analysis was performed using software at the Compute pI/Mw tool in Expasy molecular biology server (http://us.expasy.org/tools/pi_tool.html). The presence of signal peptide was analyzed by Signal P 3.0. Putative functional domains were predicted by NCBI Conserved Domain Search (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequence alignments were generated by the CLUSTAL W program (http://www.ebi.ac.uk). Phylogenetic trees were constructed with the Neighbor-joining (NJ) algorithm by MEGA version 4 (http://www.megasoftware.net/).

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2.6. Plasmid construction

Plasmid vector pcDNA4-NLRC was used to over-express JfNLRC in flounder cells. The vector was made by PCR using LA Taq polymerase (Takara Bio Inc.) and primers NLRC-F (WCUP#914), ATAGGATCCTGAGTGAGGTCACAGAGGAGGTGACGTCCT, and NLRC-R (WCUP#915), TATGAATTCTCAGAGAGACGAGGAGCCGCTGTACTCCCA, where the first and second underlines indicate *Bam*HI and *Eco*RI restriction sites, respectively. PCR conditions were; 1 min at 94 °C followed by 35 cycles of 98 °C 10 s and 68 °C 6 min, followed by final extension at 72 °C 10 min. The amplified NLRC coding region was cloned into the *Eco*RI and *Bam*HI sites of pcDNA4/HisMax expression vector (Life Technologies).

2.7. Transfection

HINAE cells derived from flounder natural embryos [18] were used for transfection, and maintained in Leibovitz's L-15 (Invitrogen Life Technologies) medium containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 250 ng/ml of amphotericin B at 20 °C. HINAE cells cultured in 12-well plates at a concentration of 1×10^6 cells/well were used for expression analysis by Q-PCR. Equal molar amounts (8.5 nM) of pcDNA4 vectors (including the empty pcDNA4 vector and pcDNA4-NLRC) were mixed with 4 μl of Lipofectamine™ 2000 and shortage DNA was occupied with pUC19 vector. The mixtures were transfected to HINAE cells in 200 μl of Opti-MEM, and then cultured at 20 °C.

After 48 h transfection, cells were infected with live *E. tarda* at 5×10^7 CFU/ml or 1×10^7 CFU/ml, and cultured at 25 °C. ED45, a virulent *E. tarda* strain [19] was used for this experiment. After wash with 3 ml of PBS five times, the cells were harvested 24 h after infection, and re-suspended in RLT plus reagent provided by the RNeasy mini kit (Qiagen) for RNA extraction. For LPS-stimulation experiments, 125 μg/ml of LPS (*E. coli* serotype 0111:B4) (Sigma) was added in the HINAE cells transfected after 48 h. The cells were harvested 24 h after the stimulation, and re-suspended in RLT plus reagent provided by the RNeasy mini kit (Qiagen) for RNA extraction as well.

2.8. Reverse transcription (RT-PCR) and quantitative real-time PCR (Q-PCR)

Prior to conventional RT-PCR, primer specific to the NACHT domain (Nacht RT:F 5′-GTCGTCTTCATCTTCGACGGTCTGGAC-3′ and Nacht RT:R 5′-CAGGAAGTGGATGTACATCTCCGTCAGGGT-3′) and a set of internal control primers, β-actin (actinF: 5′-ACTACCTCATGAAGATCCTG-3′ and actinR: 5′: TTGCTGATCCACATCTGCTG) were used. The PCR conditions were; 5 min at 95 °C followed by 30 cycles of 95 °C 30 s, 55 °C 30 s and 72 °C 30 s, followed by final extension at 72 °C 10 min. The PCR products were visualized by electrophoresis on a 1.5% agarose gel.

Quantitative real-time PCR (Q-PCR) was performed using FastStart Universal probe master (ROX) (Roche), and the StepOne Plus Real-time PCR system (Applied Biosystems) according to manufacturer's instructions. The primers used for Q-PCR were designed using Primer Express software (Applied Biosystems). Amplification efficiencies for three Q-PCR primer sets were analyzed using a previously reported protocol [20]. The primer sequences and efficiency values were following: NLRC (efficiency, 92%): WCUP-749 (Forward) TGGCGTCCGATGGTTCA and WCUP-750 (Reverse) CGTTGGACAGTTGGAGGTTTTT; IL-1β (efficiency, 93%), WCUP-377 (Forward) ATGGAATCCAAGATGGAATGC and WCUP-378 (Reverse) TTAACTCTGATGATGGATGTT; TNFα (efficiency, 95%): WCUP-47 (Forward) GTCCATCAGCCACAGGGTAT and WCUP-48 (Reverse) CGTCCTCCTGACTCTTCTGG; IFN-γ (efficiency: 100.4%): WCUP-59 (Forward) TGCAAGGATGAACAAAACCA and WCUP-60 (Reverse) AGAACTCGCCTCCTCGTACA, and β-actin (efficiency: 101%): WCUP-90 (Forward) TGATGAAGCCCAGAGCAAGA and WCUP-91 (Reverse) CTCCATGTCATCCCAGTTGGT,. The Q-PCR reactions were performed in duplicate and each contained 4 μl of diluted cDNA template (4 ng of total RNA equivalents), 5 μl of FastStart Universal probe master (ROX) (Roche), and 250 nM forward and reverse primers in a 10 μl reaction volume. The amplification steps consisted of initiation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s each followed by annealing at 60 °C for 1 min, with next a melting curve analysis step featuring 1 cycle at 95 °C for 15 s, a hold at 60 °C for 1 min, and a further hold at 95 °C for 15 s, to confirm that only a single amplicon was present. The relative expression levels for flounder NLRC and these cytokine mRNAs were determined using the flounder β-actin gene as an internal reference. The statistical *p*-values were calculated using a two-tailed paired Student's *t*-test.

3. Result and discussion

3.1. Characterization of NLR subfamily C from Japanese flounder

A partial sequence homologous to zebrafish NOD-C like receptor (GenBank accession no. XP_002661313.1) was found in the microarray results of Japanese flounder challenged with formalin-killed *S. iniae* as a transcript up-regulated with 3 times higher than that of the control [14]. Several primer sets were designed based on this partial DNA sequence for both 5′- and 3′-RACE sequencing. As a result, the full-length cDNA of the NOD-like receptor subfamily C (NLRC) was cloned and designated as Japanese flounder (JfNLRC). This transcript (GenBank acc. no. JF271924) consisted of 3976 bp with a predicted open reading frame (ORF) of 3528 bp, a 5′-untranslated region (UTR) of 87 bp and a 3′UTR of 361 bp. The ORF encoded for 1175 amino acid residues with a calculated molecular mass of 132.5 kDa and a pI of 5.3. The deduced amino acid sequence of JfNLRC was compared to other published fish NLRC and mammalian NLRC3 (Fig. 1). The NACHT domain in JfNLRC was similar to those of other fish. Three leucine-rich repeat (LRR) regions (LRR1-3) in Japanese flounder were also conserved but the other LRRs in zebrafish and mammals were not. Conserved domain analysis of JfNLR using CDD and SMART program indicated that the polypeptide was composed of a middle NACHT at amino acid residue position 347–515, three leucine-rich repeat (LRR) region at amino acid position 857–936 and the carboxyl-terminal B30.2 (PRY/SPRY) motif at amino acid position 985–1167 which resembled that of NLR (Figs. 1 and 2). Due to the absence of a signal peptide, this JfNLRC protein was predicted to be an intracellular receptor. Several types of NLR orthologs have been categorized in eukaryotes including a predominantly central NACHT and a C-terminus LRR with some extra CARD or a PYD domain in the N-terminus [3,7]. Putative functional domain organization of JfNLRC showed an extra B30.2 domain, which was similar in domain organization as has previously been observed in the zebrafish [8] and pufferfish (GenBank acc. no. XP_002661801) genomes. Homology analysis of JfNLRC with mammalian, avian and teleosts showed low homology of the full-length amino acid sequence and NB domains among NOD1, NOD2 and NLRC3 family (Table 1).

3.2. Phylogenetic tree analysis

The JfNLRC sequence showed high homology to the NLRs family rather than NALPs family following homology search analysis using the full-length cDNA (data not shown). NLR members are categorized based on the domain organization at N-terminus (Fig. 2). In zebrafish, three subfamilies of NLRs have been categorized, where

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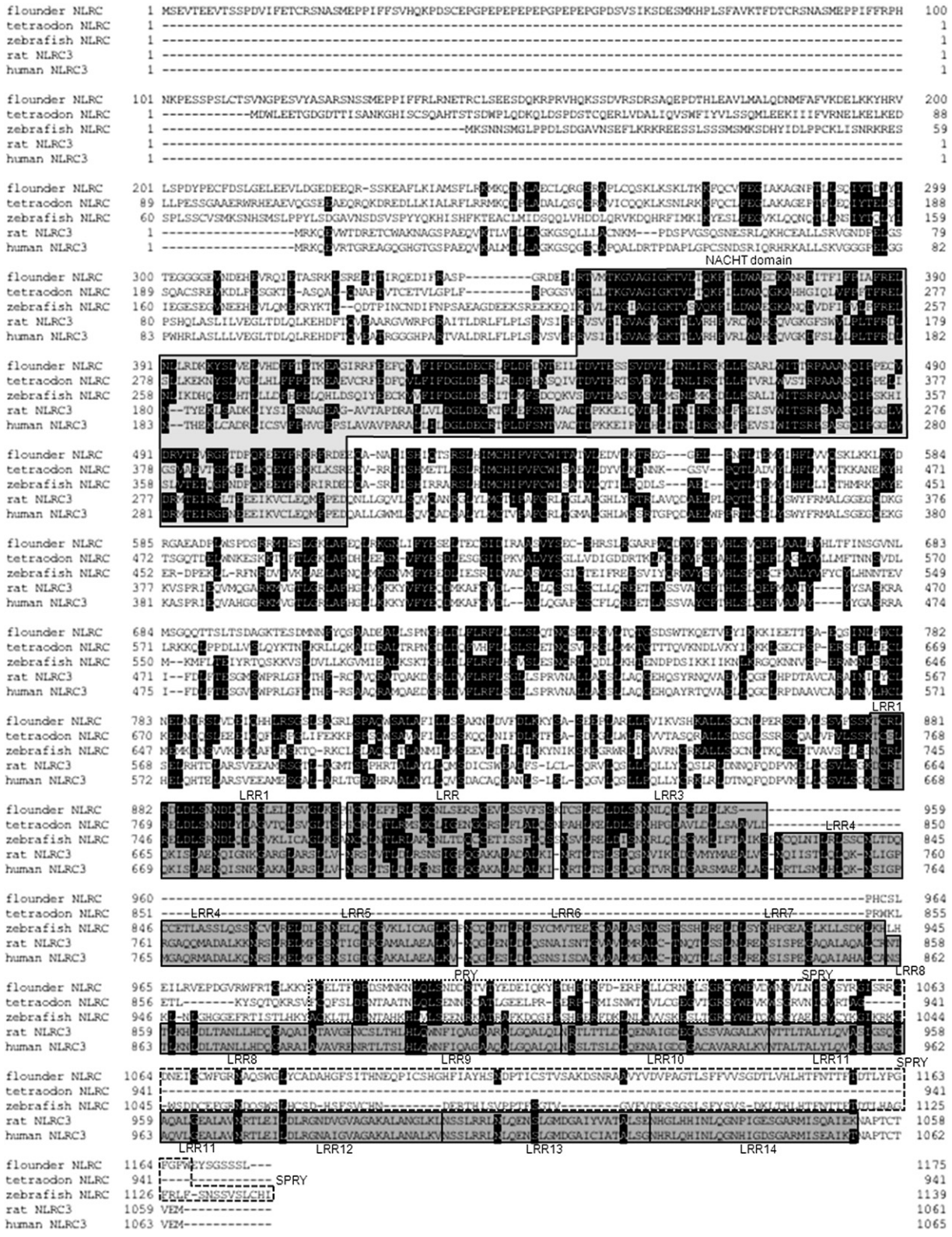

Fig. 1. Alignment of deduced amino acid sequences of Japanese flounder NLRC and other species including Japanese flounder (flounder, GenBank acc. no. JF271924), spotted-green pufferfish (tetraodon, CAG12481.1), zebrafish (NP_001107787.1), rat (XP_220212.4) and human (NP_849172.2). The predicted domains for NACHT and LRRs (LRR1-14) are indicated with gray boxes. The PRY and SPRY (B30.2) domains are indicated with boxes with dashed borders.

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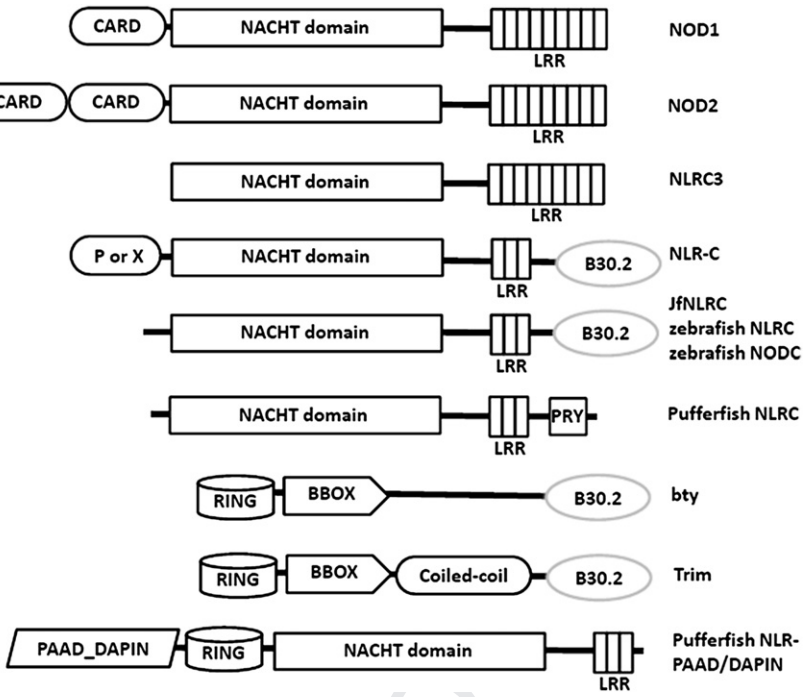

Fig. 2. Schematic diagrams depicting the domain organizations of NLRs and relative proteins. NOD1 possesses a single CARD domain, NOD3 possesses a double CARD domain, while NLRC3 (formerly NOD3) lacks this domain. Fish NLR subfamily C (NLRC) possesses middle NACHT and LRR domains which are predicted to be flanked by N- or C-terminal effector domains such as pyrin (P) or an uncharacterized effector domain (X) [8]. JfNLRC, zebrafish NLRC and zebrafish NOD-C have a C-terminal B30.2 domain while pufferfish PAAD/DAPIN have N-terminal PAAD/DAPIN and Ring domains. Other relative proteins with B30.2 domain, such as Trim and bloodthirsty (bty), play an important role in immune system.

NOD1 and NOD2 were restricted to the subfamily A [8]. However, large number of genes sharing significant homology to NLRC3 harbored distinct domains at N- or C-terminus of an original NLRC3 and therefore was classified under subfamily C (NLRC) (Fig. 2).

The phylogenetic tree of the full-length, NACHT domain, and B30.2 region of JFNLRC were constructed using the amino acids sequences from mammalian, avian and fish NLRs and TRIM proteins. As the JfNLRC possesses an extra B30.2 domain, the TRIM protein harboring a B30.2 domain was analyzed as well (Fig. 2). The phylogenetic analysis of the full-length NLRs demonstrated that JfNLRC and the fish NLR subfamily C; zebrafish NLRC, pufferfish NLRC, and pufferfish PAAD-DAPIN, were clustered together but not with other known NLRs. Furthermore, JfNLRC showed significant distance from the Trim protein cluster (Fig. 3A). By using the cluster of Trim to root the tree, all sequences can be clearly clustered according to the significant internal branch: (1) a cluster of NOD1 and NOD2, (2) a cluster of NLRC3 and (3) a cluster of fish NLRC.

Previously, the NACHT domain has been used to reconstruct the evolutionary relationships of vertebrate NACHT domain containing protein. The LRR domain has also been useful, however, it was not employed in this study due to the different repeated LRR domain of each protein making a phylogenetic analysis unsuitable [21]. Our analysis revealed that the fish NLRC subfamily C cluster shared an internal node with NLRC3 before the branch was separated to NOD1 and NOD2 clusters (Fig. 3B). Moreover, it has been revealed that the fish NLR subfamily C is restricted only to teleostei [8,21,22]. This observation supports the independent and parallel evolution among members of the NLR family [21,22].

In vertebrates, a few of the C-terminal effecter of NLRs has been reported whereas a number of NACHT protein with different C-terminal effecter such as EGF-fibrinogen, lectin_C and WD40 was observed in sea urchin and amphioxus [22,23]. The presence of a B30.2 at C-terminal of NLR was originally reported from zebrafish genome [8]. The domain composes of a subdomain SPRY incorporating a PRY domain. The evolutionary adaptation revealed that the B30.2 domain has evolved recently from an ancient SPRY domain by incorporating the PRY domain encoded by a single exon [24]. The relative protein, tripartite motif (Trim), is involved in antiviral immunity as characterized by a tripartite motif comprises of a RING finger domain, B-boxes and coiled-coil domains. Certain Trim provided an additional domain B30.2 domain (Fig. 2).

Table 1
Percentages of amino acid identity/similarity of full-length, NACHT and LRRs domains in JfNLRC and other vertebrate NOD1s, NOD2s and NLRC3s.

	Full-length	NACHT	LRRs
NOD1			
Channel catfish	21/40	26/47	34/57
Zebrafish	24/40	27/46	28/54
Chicken	21/41	27/43	34/48
Mouse	24/45	28/45	No similarity found
Human	25/44	27/44	26/35
NOD2			
Channel catfish	*Partial 22/39		30/47
Zebrafish	25/44	29/49	38/55
Mouse	25/42	30/52	31/46
Human	25/41	31/51	35/52
NLRC3			
Channel catfish	*Partial 25/42		41/70
Zebrafish	26/45	32/50	29/48
Mouse	30/49	39/58	32/47
Human	29/47	39/56	28/59
NLRC			
Zebrafish	48/62	62/78	64/77
Pufferfish	50/66	68/82	56/66
Pufferfish-PAAD/DAPIN	46/65	56/78	50/67

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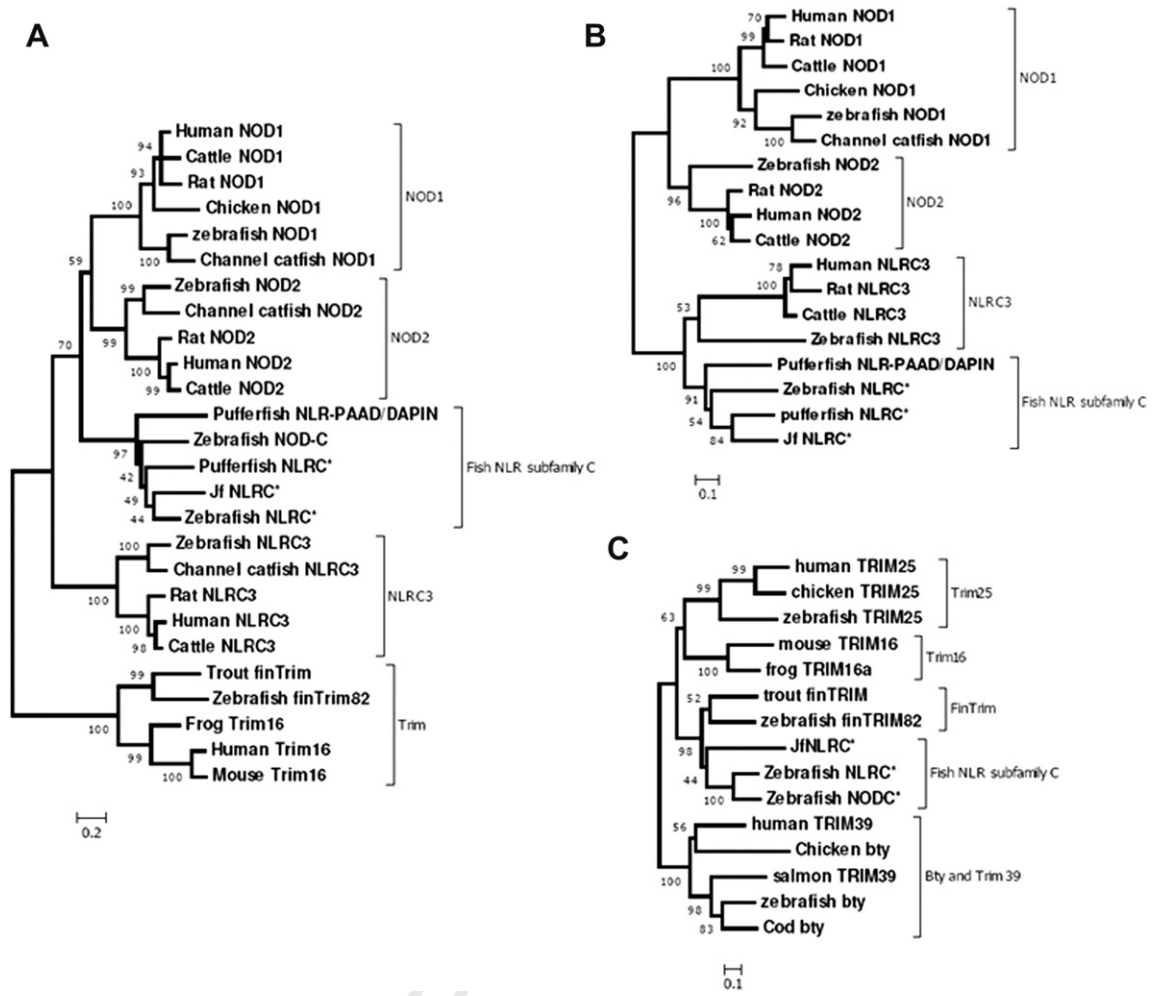

Fig. 3. JfNLRC is a representative teleost-specific NLR. Phylogenetic trees analysis (NJ, bootstrap = 1000) of NLRs and their related proteins, based on full-length (A), NACHT domain (B) and B30.2 domain (C). The numbers at the forks indicate the bootstrap. The accession numbers are follows: NOD1: NP_006083.1 (human NOD1), XP_598513.3 (cattle NOD1), NP_001102706.1 (rat NOD1), XP_418777.2 (chicken NOD1), XP_002665106.1 (zebrafish NOD1), ACM45224.1 (channel catfish NOD1), NOD2: NP_071445.1 (human NOD2), NP_001002889.1 (cattle NOD2), NP_001099642.1 (rat NOD2), XP_697924.3 (zebrafish NOD2), ACM45225.1 (channel catfish NOD2), NLRC3: NP_849172.2 (human NLRC3), XP_584462.3 (cattle NLRC3), XP_220212.4 (rat NLRC3), XP_001920433.2 (zebrafish NLRC3), ACM45226.1 (channel catfish NLRC3), fish NLRC: CAF95481.1 (pufferfish NLR PAAD/DAPIN), XP_002661313.1 (zebrafish NOD-C), NP_001107787.1 (zebrafish NLRC), CAG12481.1 (pufferfish NLRC), Trim16: NP_001086184.1 (frog Trim16), NP_006461.3 (human Trim16), AAH52821.1 (mouse Trim16), Trim25: NP_005073.2 (human Trim25), XP_415653.2 (chicken Trim25), NP_956469.1 (zebrafish Trim25), Trim39: NP_742013.1 (human Trim39), NP_001135231.1 (salmon Trim39), finTrim: NP_001153978.1 (trout finTrim), NP_001068571.1 (zebrafish finTrim82), bloodthirsty (bty): XP_424445.2 (chicken bty), NP_001018311.1 (zebrafish bty), and AAS45170.1 (cod bty).

In this study, the phylogenetic analysis of B30.2 regions from fish antiviral Trim proteins (finTrim), Trim39, a counterpart of Trim39–bloodthirsty (Bty), mammalian Trim, which is very similar to finTrim; Trim16 and Trim25, were constructed. The analysis revealed that the fish NLRCs clustered with finTrim with significant distance from Trim39 and Bty (Fig. 3C). After using Bty and Trim39 to root the tree, the cluster of finTrim/fish NLRC diverged from that of Trim16 and Trim25 clusters. The divergence of finTrim has been previously shown to be restricted to teleostei as an independent evolution and no gene orthologs were identified from chicken and frog. *Trim 16* and *Trim 25*, which are most similar to finTrim, were ancient genes, which possessed independent evolution among animals [13]. Furthermore, it is suggested that B30.2 domain in Trim is older than that of NLRs, and that exon shuffling caused a B30.2 domain to transfer from a Trim-B30.2 gene to an NLR gene [25]. Taken together, this observation indicated this exon shuffling happened early in teleost evolution. According to finTrim evolution, the presence of B30.2 domain in JfNLRC suggests that it evolve through an exon shuffling with finTrim [13].

3.3. Expression analysis of JfNLRC

The expression of JfNLRC was determined in different tissues. The transcript was detected in all tissues investigated, with highest expression in brain, gill, kidney and PBL (Fig. 4). The same pattern of NLR subfamily C mRNA expression in naïve zebrafish was also detected in its liver, intestine and spleen [8]. The very high

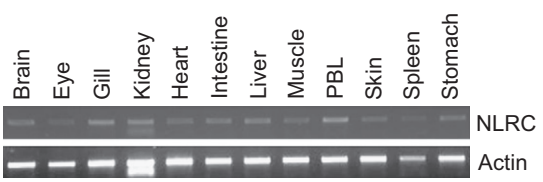

Fig. 4. Tissue distribution of Japanese flounder NLRC mRNA. Specific DNA fragments for JfNLRC transcripts were amplified by conventional RT-PCR with cDNA templates synthesized from RNAs from the indicated tissues. β-Actin mRNA was also detected as an internal control.

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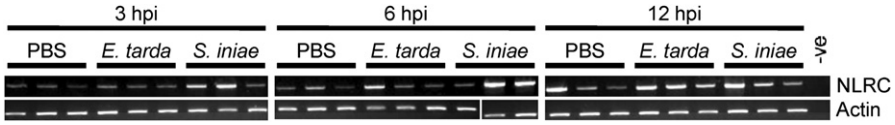

Fig. 5. Expression profiles of Japanese flounder NLRC transcripts from head kidney after Japanese flounder were treated with formalin-killed *Edwardsiella tarda* (*E. tarda*) and *Streptococcus iniae* (*S. iniae*) for 3, 6, and 12 h. Three fish were used for each treatment. β-Actin (actin) mRNA expression was also assayed as an internal control. "−ve" indicated as a negative control.

expression level of JfNLRC in gill and kidney was consistent with previous observation where the expression of genes for defense was predominant in tissues or organs exposed to the external environment or in hematopoietic tissues [26].

It is known that NLRs are involved in innate immunity, fighting against microbial invasion. Moreover, the protein containing SPRY and B30.2 domains exhibit a wide range of functions, including regulation of cytokine signaling (SOCS), RNA metabolism (DDX1, hnRNPs), intracellular calcium release (RyR receptors), immunity to retroviruses (Trim5α) as well as regulatory and developmental processes (HERC1, Ash2L). However, the functions of NLRCs have not been clearly defined in many other aspects. To investigate whether the expression of JfNLRC is responsible for immunostimulation, a time-course expression of JfNLRC following injection with FKC-killed *S. iniae* and *E. tarda* was investigated. Expression of JfNLRC was up-regulated as fast as 3 h pi in FKC-killed *S. iniae* and 6 h pi in FKC-killed *E. tarda* stimulation. The stimulation was prolonged until 12 h pi and declined thereafter (Fig. 5). The modest response against FKC-killed *S. iniae* may be due to the dose of the immunostimulant in which the cell number of FKC *S. iniae* was two-times higher than that of FKC *E. tarda*. This result suggested that the stimulation of JfNLRC is immediate against both Gram-positive and Gram-negative bacteria and indicated the acute response of JfNLRC after immunostimulation. This acute response against invading bacteria has also been proposed in growth differentiation factor 15 (GDF15) [16], goose-type lysozyme homologue (LycGL) [26] where their stimulation were quickly up-regulated within 24 h post injection. As described previously, NLRs play an important role in detecting inflammatory stimuli and mediate inflammasome assembly. The upregulation of NLRs may lead to activation of cytokines and chemokine secretion such as IL-1β and IL-18 [6,27].

Regulation of Japanese flounder NLRC gene expression by LPS-stimulation was studied *in vitro* using KL and PBL (Fig. 6). After incubation with LPS for 6–48 h, JfNLRC transcripts in KL showed increase in expression level when compared to the control. The JfNLRC transcripts were significantly up-regulated in a time-dependent manner. In contrast to this, JfNLRC transcripts in PBL showed a much smaller increase in expression level. Other fish NLRs (*i.e.* NOD1 and NOD2) such as the grass carp (*Ctenopharyngodon idella*) also show an increase in expression level after LPS-stimulation in the trunk kidney and blood [10]. Furthermore, the induction level in the trunk kidney is higher than that of the blood. The trunk kidney probably includes more cells, which respond to LPS-stimulation, than other tissues.

3.4. Induction of inflammatory cytokine gene expressions by JfNLRC over-expression

To understand the gene expression level of inflammatory cytokines (such as IL-1β and TNFα) regulated by NF-κB, which, in turn, is activated through PAMPs recognition *via* an NLR family member [27,28], IL-1β and TNFα gene expression levels were measured after LPS-stimulation or bacterial infection in JfNLRC-transfected HINAE cells. Furthermore, since an NLR family member (NOD2) in rainbow trout induces interferon gene expression, especially IFN-γ [29], IFN-γ gene expression was also examined. TNFα gene expression was induced by LPS in HINAE cells transfected with empty constructs although IFN-γ and IL-1β transcripts didn't show sufficient increase in expression in the control cells (Fig. 7A). In JfNLRC-over-expressing cells, the gene expressions of IFN-γ, IL-1β and TNFα were significantly induced by LPS-stimulation (Fig. 7A). IL-1β showed the greatest increase in expression level (3.5 fold) when compared to pcDNA-empty control in LPS-stimulated HINAE cells. These results suggest that JfNLRC is involved in the induction of IL-1β expression after LPS-stimulation. A PRR that recognizes LPS has never been found in teleosts. Zebrafish TLR4 does not recognize LPS and attenuates NF-κB activation [30] although mammalian TLR4 recognizes LPS mediated by NF-κB activation [31]. NLRC in teleosts could be the molecule that enhances NF-κB activation upon LPS-stimulation. However, further experiments are necessary to confirm this idea.

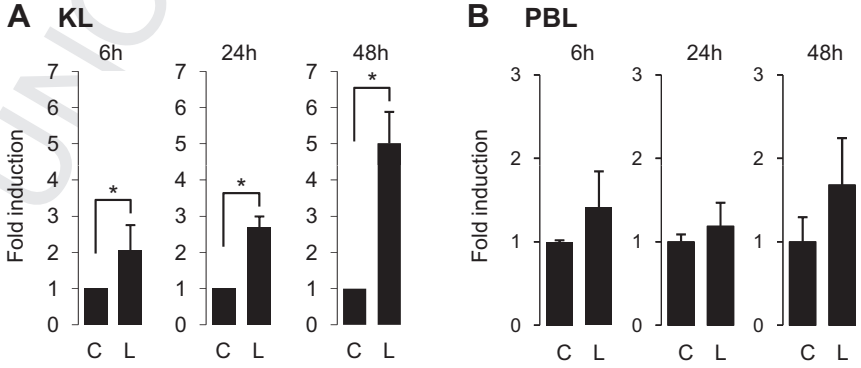

Fig. 6. *In vitro* gene expression of Japanese flounder NLRC in the LPS-stimulated leukocytes. (A) Whole kidney leukocytes (KL) and peripheral blood leukocytes (PBL) isolated from three fish were stimulated with LPS (250 μg/ml) for 6, 24 and 48 h. Total RNA was extracted from these cells, and the synthesized cDNA was used for Q-PCR. Expression levels are shown relative to the control at the same time point. Error bars indicate the standard error (SEM) for the results of three individual fish. Student's *t*-test was used for statistical analysis, and asterisks indicate significant differences between control (C) and LPS-treated (L) leukocytes of each time point (6, 24 and 48 h) ($^{*}p < 0.05$; $^{**}p < 0.01$).

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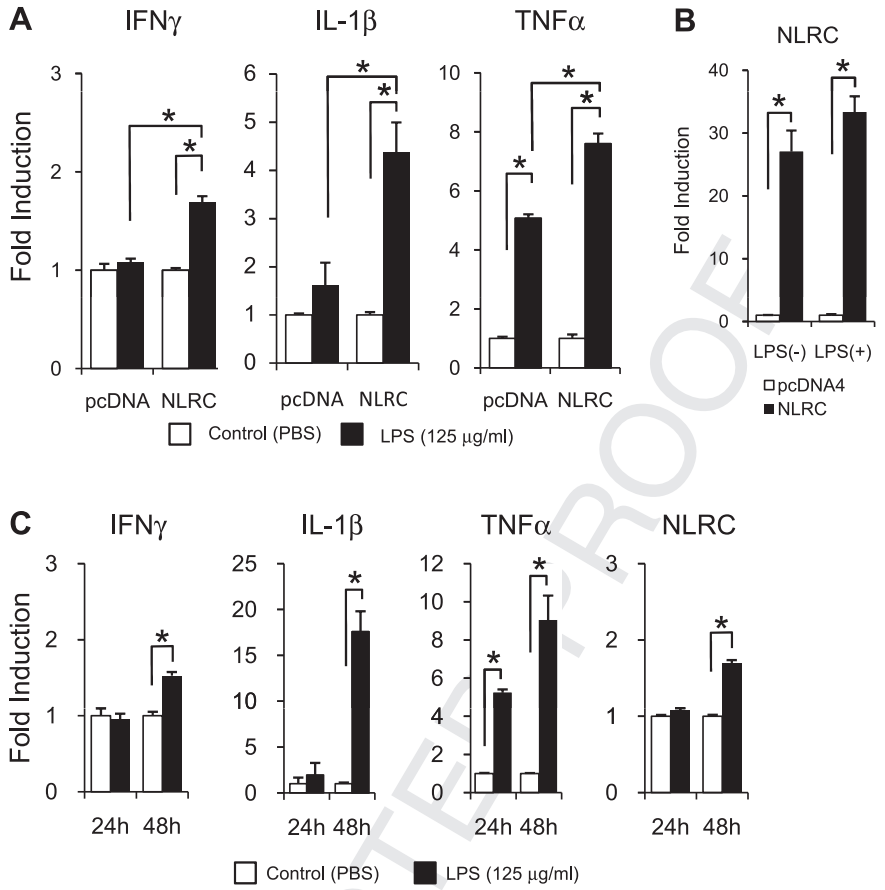

Fig. 7. Expressions of IFN-γ, IL-1β and TNFα in JfNLRC-transfected HINAE cells stimulated with LPS (A). An expression plasmid encoding full-length JfNLRC or pcDNA4 (as a negative control) was transfected into HINAE cells. Forty-eight hours after transfection, cells were stimulated with LPS (125 μg/ml) and incubated at 20 °C for 24 h. The gene expression of IFN-γ, IL-1β and TNFα were examined by Q-PCR. (B) JfNLRC expression was also measured, and the transcripts were significantly induced in the JfNLRC-transfected cells. The β-actin gene was used as an internal control to normalize the cDNA template. Expression levels are shown relative (fold increase) to the non-stimulated pcDNA-empty or pcDNA-NLRC transfected HINAE cells (control). (C) Expressions of IFN-γ, IL-1β, TNFα and JfNLRC in non-transfected HINAE cells stimulated with LPS for 24 and 48 h were examined. Error bars indicate the standard error for the results of three individual fish. Student's *t*-test was used for statistical analysis, and asterisks indicate significant differences ($^*p < 0.01$).

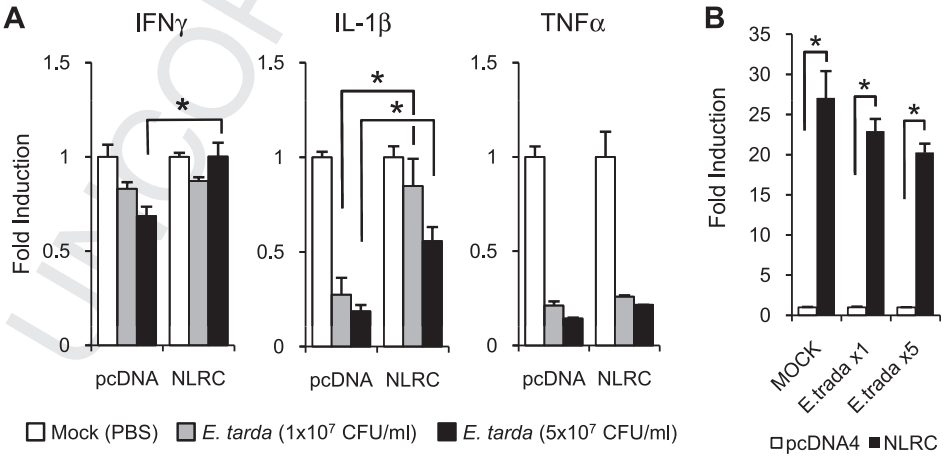

Fig. 8. Expressions of IFN-γ, IL-1β and TNFα in JfNLRC-transfected HINAE cells infected with *E. tarda* (A). An expression plasmid encoding full-length JfNLRC or pcDNA4 (as a negative control) was transfected into HINAE cells. Cells were incubated for 48 h (at 20 °C) after transfection, infected with 1×10^7 CFU/ml or 5×10^7 CFU/ml of *E. tarda* (ED45 strain) and incubated at 25 °C for 24 h. The gene expression of IFN-γ, IL-1β and TNFα were examined by Q-PCR. The β-actin gene was used as an internal control to normalize the cDNA template. Expression levels are shown relative (fold increase) to the non-stimulated pcDNA-empty or pcDNA-NLRC transfected HINAE cells (control). (B) JfNLRC expression was also measured by Q-PCR. The JfNLRC expression was significantly induced in the JfNLRC-transfected cells. Abbreviations of *E. tarda* x1 and *E. tarda* x5 indicate 1×10^7 CFU/ml and 5×10^7 CFU/ml of *E. tarda*, respectively. Error bars indicate the standard error for the results of three individual fish. Student's *t*-test was used for statistical analysis, and asterisks indicate significant differences ($^*p < 0.01$).

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In non-transfected HINAE cells stimulated with LPS, IFN-γ, IL-1β and TNFα genes showed increase in expression 48 h post-stimulation, but IFN-γ and IL-1β genes were not after 24 h (Fig. 7C). JfNLRC expression was also weak. In contrast, TNFα gene expression was also induced after 24 h in the non-transfected and LPS-stimulated HINAE cells. These results suggest that IFN-γ and IL-1β expressions were weakly or slowly induced in the HINAE cells after LPS-stimulation. These evidences support the reason that IFN-γ and IL-1β genes doesn't show increase in expression by LPS-stimulation in the pcDNA-transfected HINAE cells in Fig. 7A.

Exposure of HINAE cells to *E. tarda* for 24 h reduced the expression of all examined genes (Fig. 8). However, in HINAE cells infected with *E. tarda*, IL-1β mRNA in the JfNLRC-over-expressing cells showed approximately 2- to 3-fold increase in expression when compared to pcDNA4-empty control, while IFN-γ gene expression was slightly induced and TNFα expression wasn't induced (Fig. 8A). In HINAE cells transfected with NLRC, lowering the concentration of *E. tarda* increased the induction level of IL-1β gene expression but decreased that of IFN-γ. In zebrafish, gene expression of IL-1β and TNFα is induced by live *E. tarda* infection when compared to healthy fish [32]. IFN-γ gene expression is also induced by live *E. tarda* (avirulent vaccine) [33]. However, the results in this study showed that these cytokines expression levels were lower compared to the control. Iron-cofactored superoxide dismutase of *E. tarda* inhibits macrophage-mediated innate immune response in Japanese flounder [34]. In HINAE cells, a virulence factor of *E. tarda* could inhibit the activation of NLRC pathway or/and the expression of these cytokines. In contract, the present results (Fig. 8) indicate that this inhibition could be prevented by over-expression of JfNLRC. However, further studies are needed to determine whether JfNLRC can recognize *E. tarda*, and to elucidate the mechanism by which the JfNLRC-mediated host defense interacts with bacterial infection.

In conclusion, we cloned the full-length nucleotide-oligomerization domain (NOD)-like receptor (NLR) belonging to the subfamily C from Japanese flounder. This receptor (JfNLRC) is structurally and phylogenetically closely related to zebrafish and pufferfish NLRCs. JfNLRC was highly expressed in immune-related tissues and was immediately stimulated by LPS and by FKC-killed *S. iniae* and *E. tarda*, suggesting that JfNLRC has an important role in bacterial infection and innate immunity. Furthermore, the gene expression of IL-1β was clearly induced in JfNLRC-over-expressing HINAE cells after LPS-stimulation and bacterial infection. These results suggest that JfNLRC is a molecule specific to teleosts that induces IL-1β gene expression mediated *via* LPS-stimulation.

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